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Molecular Analysis of *Alternaria* Populations Early Blight Causal Agents in Potato Plants

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Economic Losses Caused by Potato Early Blight

Country	Yield losses	Source
USA (Colorado)	10-57 %	Harrison et al (1965)
USA	20-30 %	Johnson et al. (1986), Fry (1994)
Russia	25%, locally 60 %	Reinoch (1974)
Netherlands	Up to 50 %	Hadders (2002)
Brasil	Up to 73 %	Brune et al. (1998)
South Africa	Up to 50 %	Denner & Theron (1999)
Poland	10-32 % 6-45 %	Kuczynska (1992) Kapsa & Osowski (2004)
Germany	10-30 %	Hausladen (2005)

Although losses may exceed 20 % in Europe, intensive fungicide uses normally keep damages at levels below 5 %

Recent Trends in Fungicide Protection of Potato Crop in Europe

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- Regulatory restrictions of Early blight effective compounds
  - **Ban of organotin compounds in late 90's**
  - **Limitation of mancozeb uses on potato**

- Changes in the populations of *Phytophthora infestans* have an indirect impact on Early blight epidemics
  - **Development of highly aggressive populations of *P. infestans* (Blue A2_13 strains)**
  - **More specific Late blight fungicides are required**
  - **Late blight fungicides recently introduced on the market have no or limited Early blight activity (e.g. fluazinam, cyazofamid, fluopicolide, mandipropamid)**

Need to adapt fungicide strategies to the new context.

Efficacy of Fungicides

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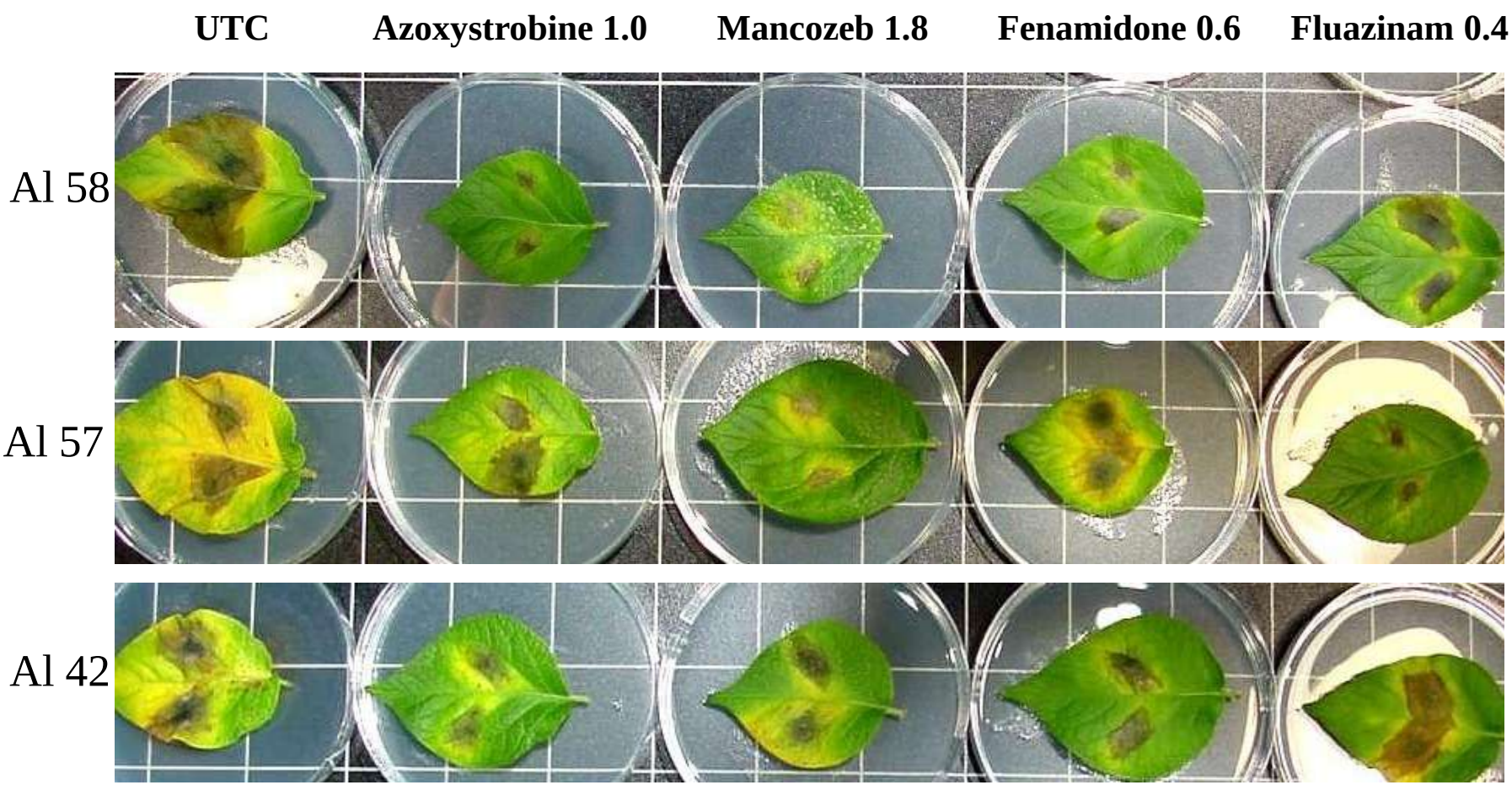

Fungicide strain dependant response and resistance to complex III QoI.

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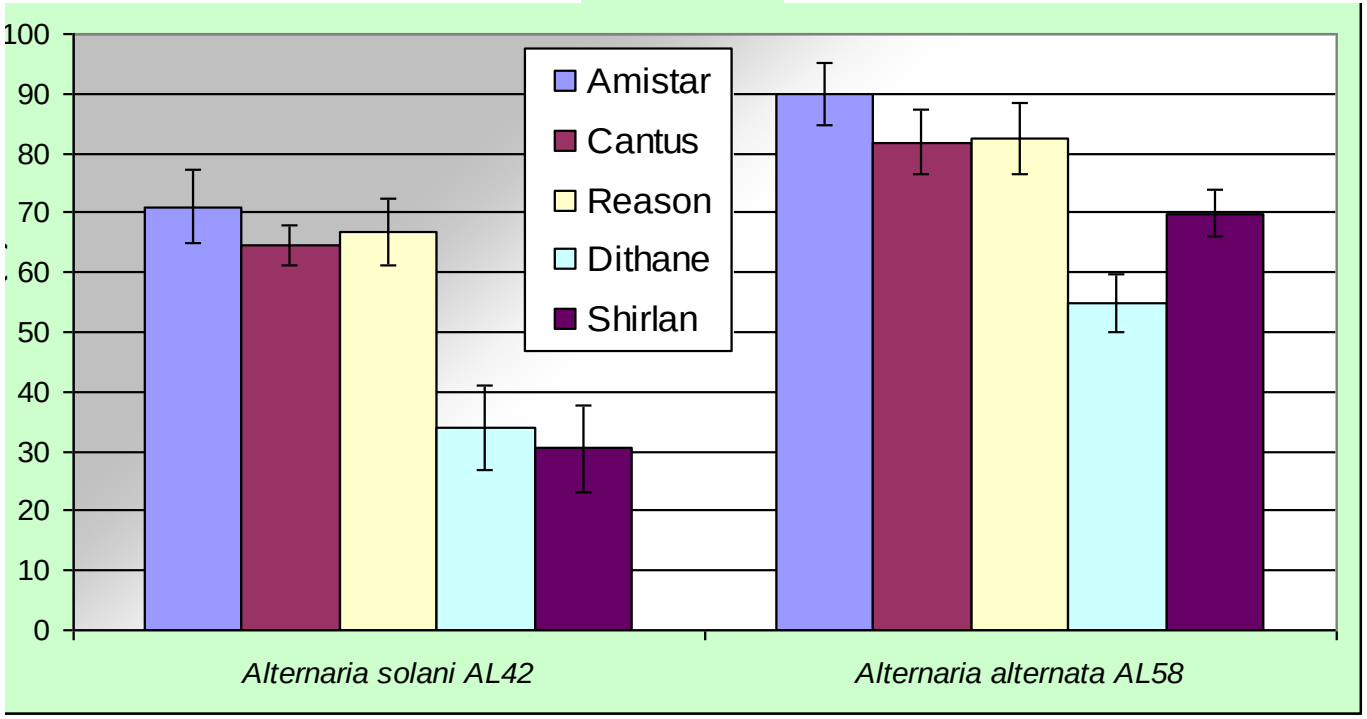
Efficacy of Fungicides



Since the efficacy of treatments can vary in function of the strains and species a accurate diagnostic is needed.

Alternaria sp. complex

ALTERNARIA solani ⟷ **ALTERNARIA alternata**

At the moment, it is not possible to distinguish between necrotic spots caused by *A. solani* or by *A. alternata* from visual observation of symptoms

Alternaria sp. Biological Diagnostic Based on Spore Morphology

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A. alternata *A. solani*





- **All the strains are not able to sporulate *in vitro***
- **Damaged or dead samples cannot be analyzed**
- **When associated with *Alternaria alternata*, *A. solani* sporulates later on the artificial media tested.**

Fungus isolation from leaf spots and microscopy observations of spores is fastidious and could lead to wrong conclusions

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Correlation Between Field and Conidia Diagnostic

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Sample code	Expectation/ Field symptôms	Conidia diagnostic
07GE049		*alternata*
07GE051 A		No spores
07NL020		No spores
07PL008		*alternata*
07PL009		*alternata*
07PL010		*alternata*
07PL011		*alternata*
07PL059A		*alternata*
07PL059B		*solani*
07PL060B		*alternata*
07NL024	*solani*	*alternata*
07NL029	*solani*	*alternata*
08GE028	*solani*	*alternata*
08BE041	*solani*	*solani*
08GE029	*alternata*	*alternata*

Molecular diagnostic using PCR could reveal mix populations or can be used to analyzed dead field samples

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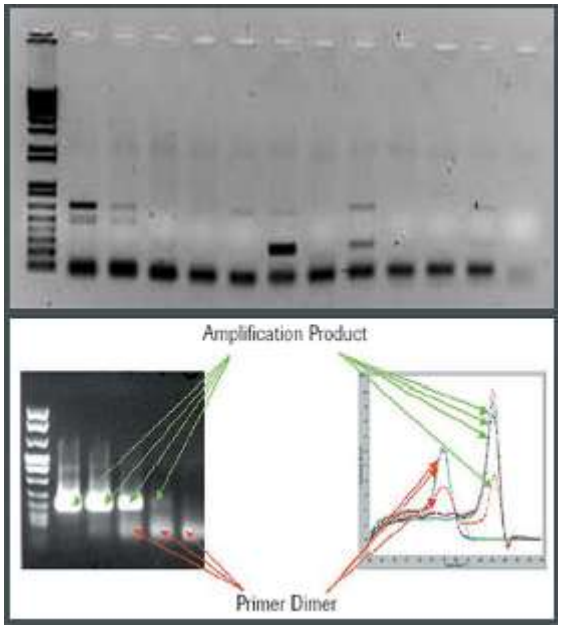
Alternaria ssp. Genotyping

- PCR-technique is the most appropriate way to distinguish Alternaria species from leaf samples collected in the field

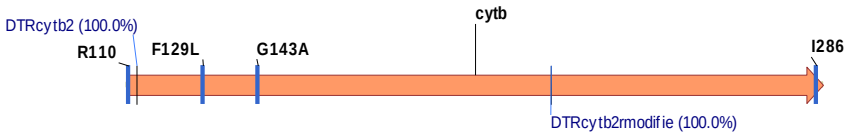


- PCR (polymerase chain reaction) can:
  - differentiate between *A. solani* and *A. alternata* species by generating specific amplicons
  - quantify the frequency of given genes (*e.g.* resistance to fungicides)

- The gene *CytB* from complex III of respiratory chain to differenciate the species by qPCR was choosen for two major reasons:
  - **An important difference in the organisation of this gene between these two species (number of introns is different)**
  - **Mutations of this gene confer a resistance to strobilurins (QoI of complex III)**

Known Gene Structures

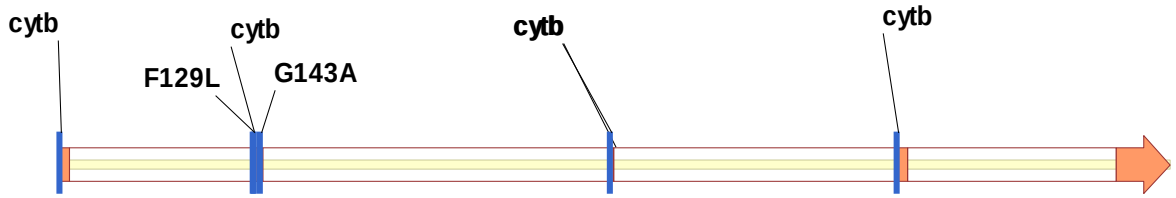
- *A. alternata*:
  - No intron described
  - The mutations confering resistance are localized on the same exon



DQ209283 A alternata gb gDNA

534 bp

- *A. solani*:
  - 5 exons and 4 introns were shown.
  - The mutation confering resistance are localized on the same exon



Alternaria solani DQ209284 gb gDNA

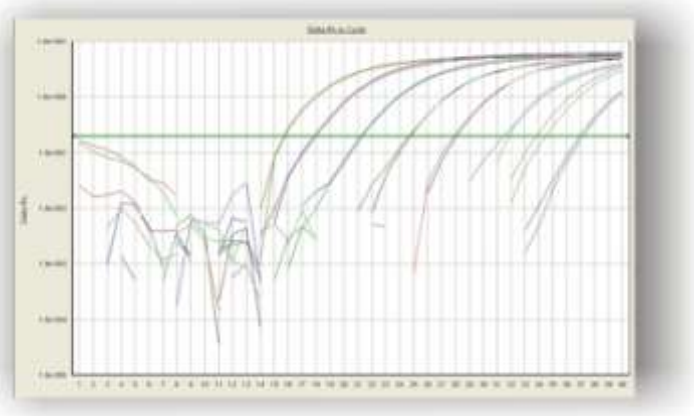
6844 bp

- High homology between both coding sequences

Grasso *et al.* 2006

Project

- A region of the gene *CytB* from strains of both species, from different geographical area, were cloned and sequenced



- qPCR primers to distinguish the genes between the two species were designed
- The primers were tested on different strains grown *in vitro* and on infected leaves to distinguish both species

Alternaria alternata Sequencing

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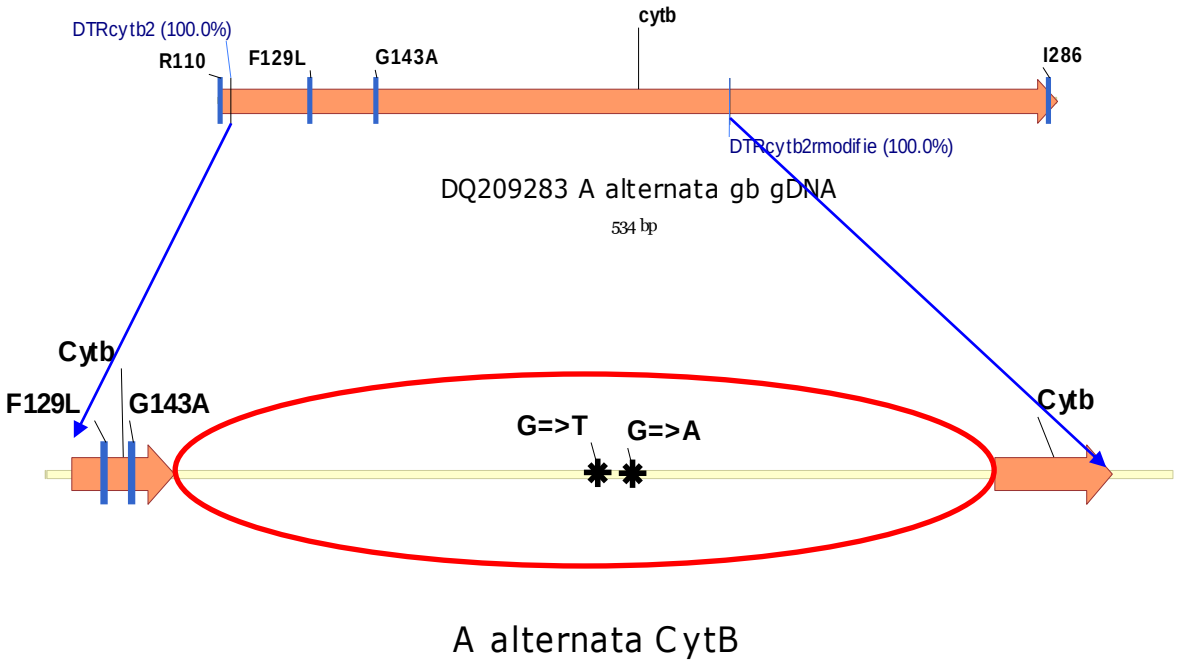
After the sequencing of 5 European strains an intron, not described in the literature was found in the sequence of the *CytB* gene.

The two mutations conferring the resistance to QoI inhibitors (strobilurine) (F129L and G143A) are still localized on the same exon



A alternata CytB

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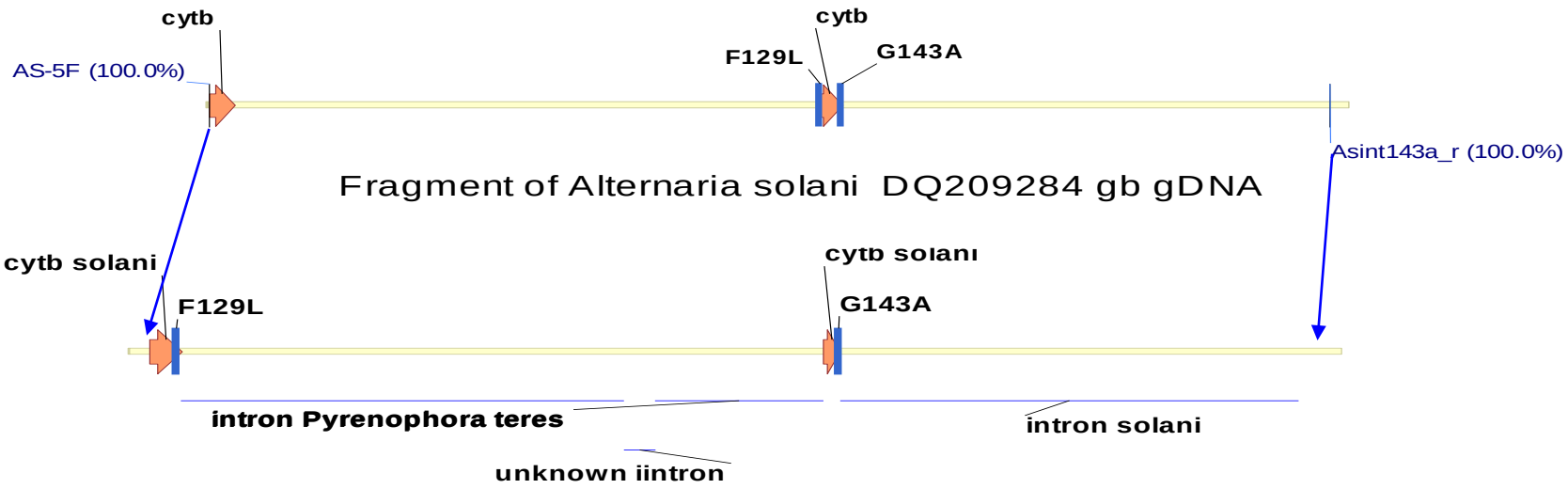
Alternaria solani Sequencing

After sequencing of two strains from USA and 4 strains from Europe we found:

- **The gene structure of the two European strains were similar with the already published one**
- **The gene structure of the two other european strains and the American strains were different:**
  - The first intron differs (*Pyrenophora teres* like intron instead of *A. solani* intron)
  - The Exon organisation was found different
  - The two mutations were separated by the intron, and the exon sizes were differents



A. alternata q-PCR Primer Design

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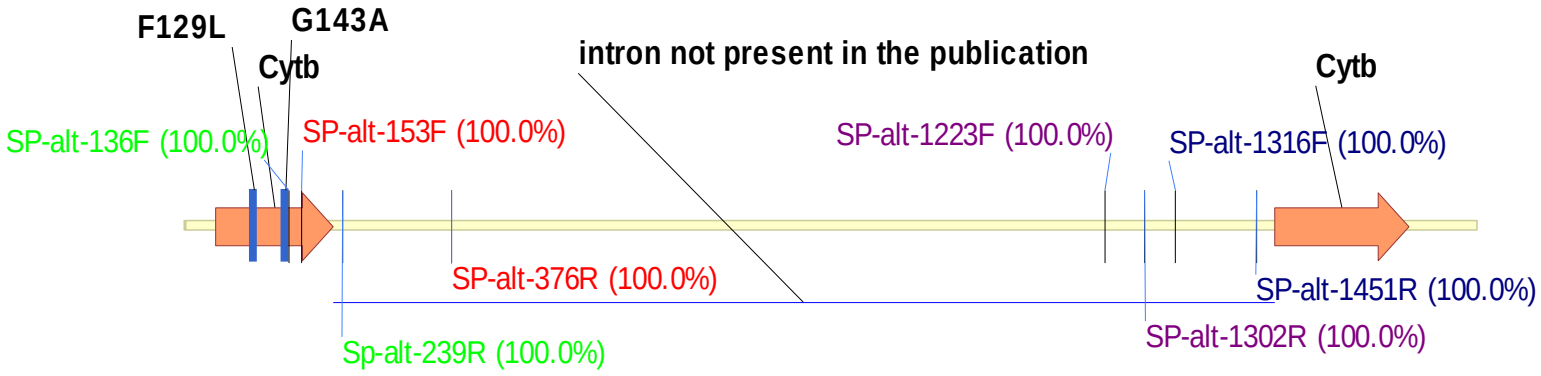

Sp-alt1 concentration F300-R50 efficacy 1,95 with R^2=0.98 (delta Ct with solani ~10)

SP-alt2 concentration F300-R50 efficacy 1,85 but amplified *A. solani*

SP-alt3 concentration F900-R50 efficacy 1,8 with R^2=0,99 (delta ct with solani ~8)

SPalt4 concentration F300-R900 efficacy 1,43 with R^2=0,97 (delta Ct with solani >15?)

SP alt1 ,3 and 4 were tested on all strains of *A. alternata* and *solani*. For SPalt4 the number of cycle need to be increased to determine the specificity.

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A. solani q-PCR Primer Design

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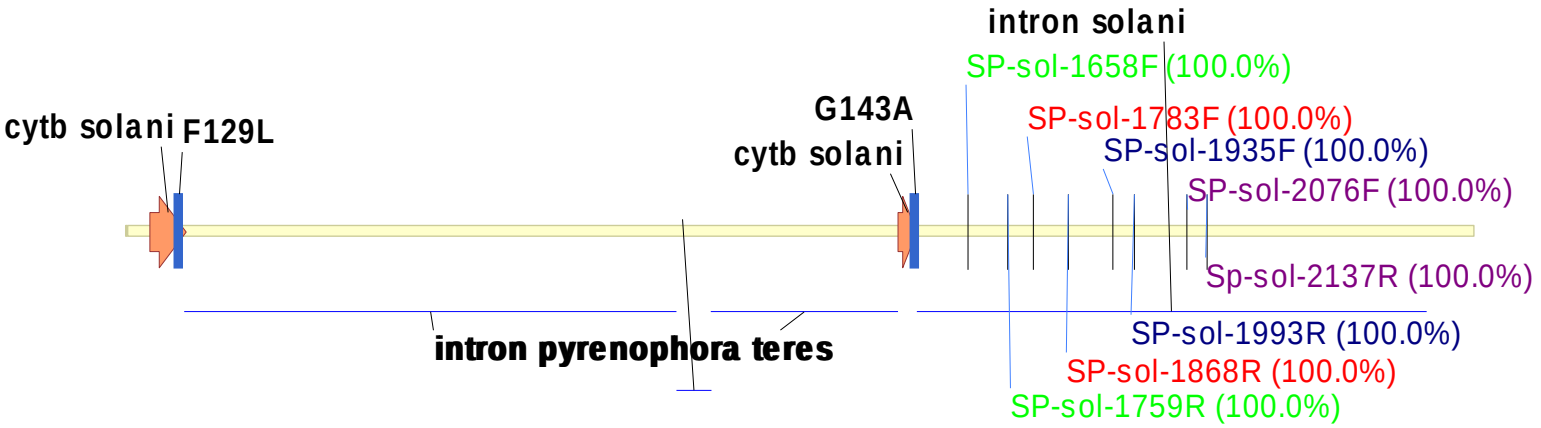

Alternaria solani (american strain)

2574 bp

SPsol1 good effecacy but not strain specific

SPsol2 good efficacy but not strain specific

SPsol3 concentration F900-R50,

efficacy 1,78 with R^2=0,96 (delta Ct with *A. alternata* >10)

SPsol4 good efficacy but not strain specific

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Test of Infected Leaves

- To confirm the accuracy of the selected primers, test of infected leaves were performed
- Leaf disks infected with spores of *A. alternata* (100 000spores/ml) or *A. solani* (50 000spores/ml), or non infected (control) were used
- 10 disks representing different ratio of both species were used for each assay:
  - 10 disks of infected leaves by *A. solani* + 0 disks of infected leaves by *A. alternata*
  - 9 disks of infected leaves by *A. solani* + 1 disks of infected leaves by *A. alternata*
  - 8 disks of infected leaves by *A. solani* + 2 disks of infected leaves by *A. alternata*
  - ……
  - 0 disks of infected leaves by *A. solani* + 10 disks of infected leaves by *A. alternata*
  - 10 disks of non infected leaves

- The disks were lyophilised, grinded and total DNA was extracted by miniprep Qiagen kit

qPCR on Infected Leaf Extracts

- dilutions 1/10, 1/100 and 1/1000 were tested.
- The qPCR was done on the ABI prism7900HT (96 wells), the standard dilution of gDNA of *A. solani* and *alternata*, the DNA extracted from non infected leaves was added as control.
- After the run, analysis was done with the SDS software

q PCR Data (1)

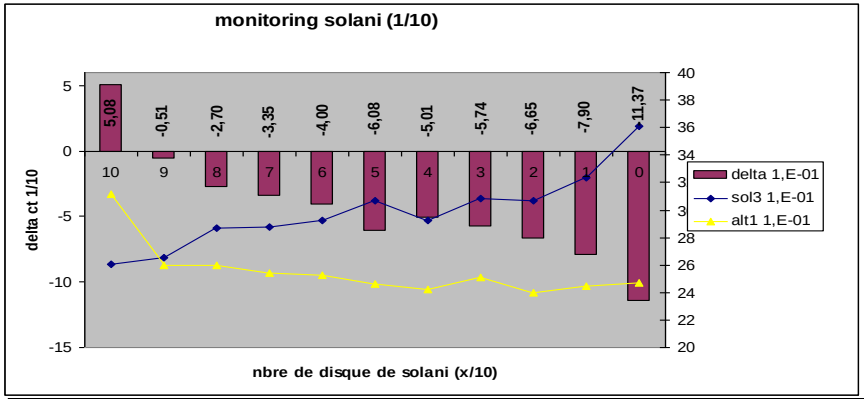
- Control (non infected leaf):
  - ALT1 give a Ct around 31 with non infected tissues and the Ct is constant with the different dilutions (Δ2between dilution 1/10 and 1/1000)
  - SOL3 give a Ct around 37 with plant non infected and is increased with the different dilutions (Δ6 between dilution 1/10 and 1/1000)

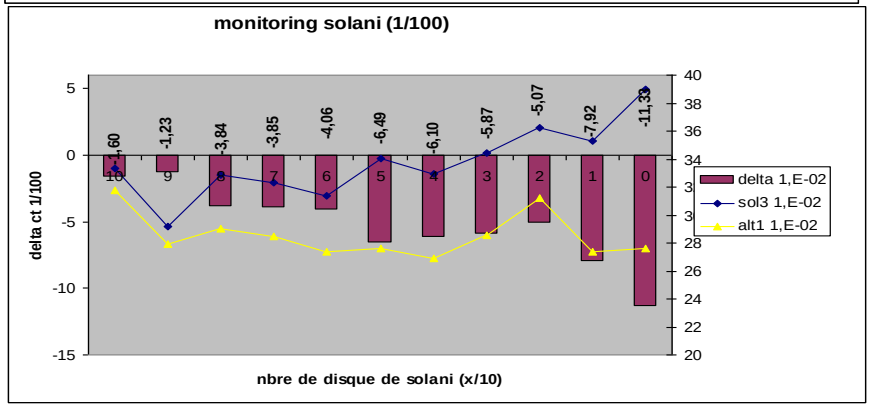
- ΔCTs and DNA% with the standard curve were calculated:
  - With ALT1 a small quantity of *A. alternata* is enough to detect an amplification and then the Ct didn't change significantly (Ct difference is 2 between1 disk and 10 disks)
  - With SOL3 the difference is more important: 6 between 1 and 10 disks

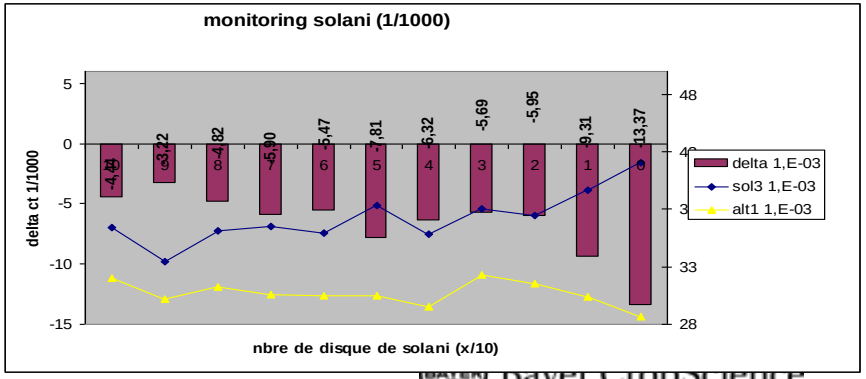
q PCR Data (2)

- With the dilution 1/10 the delta Ct increases more than with the other dilutions (more difference between each ratio)
- The DNA% shows that the % of *A. alternata* is more important than the % of *A. solani* in one disk

disks mix		1/10	
nb alt disk	nb sol disk	DNA %alt	DNA %sol
0	10	0	100
1	9	30	70
2	8	56	44
3	7	65	35
4	6	72	28
5	5	82	18
6	4	86	14
7	3	88	12
8	2	91	9
9	1	95	5
10	0	100	0







Example of Field Sample Diagnostic

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Sample code	Expectation/ Field symptôms	Conidia diagnostic	Molecular diagnostic
07GE049		*alternata*	*50%alt-50%sol*
07GE051 A		*No spores*	*50%alt-50%sol*
07NL020		*No spores*	*93%alt-10%sol*
07NL024	solani	*alternata*	*100%alt*
07NL029	solani	*alternata*	*100%alt*
07PL008		*alternata*	*50%alt-50%sol*
07PL009		*alternata*	*85%alt-15%sol*
07PL010		*alternata*	*100%alt*
07PL011		*alternata*	*100%alt*
07PL059A		*alternata*	*100%alt*
07PL059B		*solani*	*14%alt-86%sol*
07PL060B		*alternata*	*80%alt-20%sol*
08PL027	solani	*alternata*	-
08GE028	solani	*alternata*	*100% solani*
08GE029	*alternata*	*alternata*	*95% alternata – 5%solani*
08BE041	solani	solani	*100% solani*
08GE029	*alternata*	*alternata*	*95% alternata – 5%solani*

Molecular diagnostic using PCR is revealing mix populations or can be used to analyzed dead field samples

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Conclusions

- On a mix of leaf disks, the presence of both species (*A. solani* alone, *A. alternata* alone or a mix) can be detected
- The analysis can be done by the analysis of the delta Ct or the percentage of DNA (calculated with a standard)
- The limit of detection is that given by the Ct found with non infected plants
- qPCR based diagnostic offers a robust technology for a fast and reliable to quantitatively analysis of field samples to:
  - distinguish the two “species”
  - amplify the region containing the mutations F129L and G143A for direct determination by the GeXP technology
- A novel sub-species of *A. solani* was characterized suggesting possible horizontal gene transfer between *P. teres* and *A. solani*

A combination of PCR and GeXP will offer the possibility to perform genotyping and SNPs analysis fast and in a high throughput

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Thank you very much for your kind attention.